"Isolation and Characterization of Concanavalin A from the seeds of *Canavalia ensiformis*"

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CERTIFICATE

This is to certify that the thesis entitled "**Isolation and Characterization of Concanavalin A from the seeds of *Canavalia ensiformis***" which is being submitted by Ms Rekha Marndi, Roll No. 410LS2057, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I, Rekha Marndi, hereby declare that this project report entitled **"Isolation and Characterization of Concanavalin A from the seeds of *Canavalia ensiformis*"** is the original work carried out by me under the supervision of Dr. Sujit K. Bhutia, Assistant Professor, Department of Life Science, National Institute of Technology Rourkela (NITR) and to the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

(Rekha Marndi)

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And to all mighty, who made all things possible……..

(REKHA MARNDI)

CONTENTS

1. ABSTRACT

Concanavalin A was isolated from Jackbean (*Canavalia ensiformis*) seeds. The protein was purified by using affinity chromatography. The activity of the Con A was determined by haemagglutinin assay and the purity of the protein was tested by Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The activity of lectins is quantified by their ability to agglutinate erythrocyte. The eluted protein exhibited agglutinating activity when reacted against different types of fresh erythrocytes. In SDS-PAGE, it has been concluded that the molecular weight of Concanavalin A is 104 kDa. It is a homotetramer in which transition metals (Magnesium or Calcium ions) are bound to each subunit that helps this lectin to bind to α-mannose or α-galactose in sugars.

INTRODUCTION

2. INTRODUCTION

Plant lectins are the proteins that specifically bind to carbohydrates and recognize different structures of sugars. Different kinds of lectins have different structures, sequences and affinities for different carbohydrates. Their biological functions include cell to cell interaction, host pathogen interactions and innate immune response. Lectins are mostly found in leaves, vegetative tissues, and roots and in seed cotyledon. These are the glycoproteins that specifically bind to mono or oligosaccharide (Peumans and Damme, 1995) without altering their covalent structure. They are usually of originated from plants mostly found in some leguminous seeds. They form 3% of the total weight of mature seeds. Mainly, legume lectins form the basic model to study them in molecular form (Barondes, 1988). The crystal structures of the lectin-carbohydrate complex forms the basic model to study the interactions of carbohydrates and proteins (Sumner *et al*, 2002). Although lectins occur in living organisms, plant lectins were the first proteins to be studied. 42% of the 3D structures of lectin are obtained from plants and the rest are obtained from animals, bacteria, fungi, algae and viruses. Mostly, the largest and the best family of plant lectins are obtained from the leguminous seeds (Goldstein and Poretz, 1986).They have four sugar binding pockets are present towards the four corners of a tetrahederon so that the binding sites are at a maximum distance from each other.

2.1 Types of lectin

Structurally, the mature lectins are divided into

a) Merolectins,
b) Hololectins,
c) Chimerolectins,
d) Superlectins.

a) Merolectins

They are monovalent with the single - carbohydrate binding domain. So, they cannot precipitate glycoconjugates or agglutinate cells. e.g., Hevein, is a chitin binding protein (Van Parijs *et al*, 1991) and is obtained from latex of rubber tree.

b) Hololectins

They contain two homologous carbohydrate binding domains that can bind to same or structurally similar sugars. This lectin is divalent or multivalent. Hence, they agglutinate cells or precipitate glycoconjugates (Van Damme *et al*, 1998).

c) Chimerolectins

These are the fusion proteins with multi carbohydrate – binding domains. They can behave as merolectins or hololectins depending upon the number of carbohydrate binding domains.

d) Superlectins

They have atleast two carbohydrate binding domains that recognize structurally unrelated sugars. These are the special group of chimerolectins with two different carbohydrate-binding domains that are tandemly arrayed structurally and functionally.

On the basis of molecular structure, lectins are classified into 3 different types:

a) Simple lectins,
b) Multi-domain lectins,
c) Macromolecular assemblies.

a) Simple lectins

It consists of a few subunits of molecular weight 40 kDa or less. It may contain an additional domain other than carbohydrate binding sites. e.g., Galectins (S-lectins).

b) Multi-domain lectins

These are the composite molecules with a wide range of molecular weights, having several kinds of domains. Among them, only one domain has a carbohydrate binding site. These domains include diverse proteins from viral hemagglutinins and animal lectins. These lectins are monovalent but they are embedded in the membranes, so they act in multivalent function.

c) Macromolecular assemblies

They are found in bacteria in the form of pilli. These are filamentous, heteropolymeric organelles consisting of different types of sub-units arranged helically. The filamental shaft is made up of major sub-unit polymers that play a structural role (Ofek and Sharon, 1990; Ofek and Doyle, 1994;

Gaastra and Svennerholm, 1996). But in minor component of the pilli, only one subunit has a carbohydrate binding site that help in binding and recognizing specific sugar of the pilli.

2.2 Plant lectins

Plant lectins are the complex and heterogenous group of proteins (Van Damme *et al*, 1998) because their molecular structure, biochemical properties and carbohydrate-binding specificity is different. Beside seeds, plant lectins are also found in all vegetative tissues like leaves, bark, stems, rhizomes, bulbs, tubers, etc. (Etzler, 1985 and 1992; Peumans and Van Damme, 1998 and 1999). In seed, lectins constitute 1-10% or even 50% of the total seed proteins and 1-20% of the total vegetative proteins (Peumans and Van Damme, 1998).

Based on the amino acid sequence, plant lectins are sub-divided into 3 different types. These include:

a) Legume lectins,
b) Monocot mannose-binding lectins,
c) Chitin-binding lectins,

a) Legume lectins

They comprise the largest family of the plant lectins belonging to the family-Leguminosae. Most of the work in the field of biochemistry, physiology and molecular biology has been carried out with these lectins. The first plant lectin to be purified and crystallized was Concanavalin A (Sumner and Howell, 1936) and its primary and three-dimensional structure was determined (Edelman *et al*, 1972; Hardman and Ainsworth, 1972). The first plant lectin gene that was sequenced was soyabean seed lectin (Vodkin *et al*, 1983).

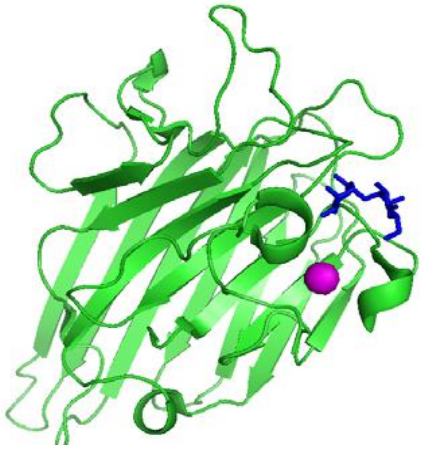
Mostly, the legume seed lectins are found in the storage protein vacuoles (Etzler, 1986). Several legume consists of two or more different seed lectins, e.g., *Phaseolus vulgaris*. Some legume lectins are found in the vegetative tissues like leaves, stems, bark, roots and root nodules e.g., bark lectins from *Robinia pseudoacacia*, leaf lectins from *Griffonia simplicifolia* (Van Damme *et al*, 1998).

These lectins occur in dimeric or trimeric forms, each subunit (25-30 kDa) having one carbohydrate binding sites. Most of these lectins are single-cell proteins. e.g., Concanavalin A.

Structure

In the 3D structure of lectin, the front face of the monomer is built up of a curved seven stranded β-sheet and back face is made up of a flat six stranded β-sheets (fig.1). Both the front and the back face are inter-connected by turns and loops to form a flattened dome shaped structure or jelly roll motif. Four loops are located at the upper part of the dome forming the binding site for the monosaccharide (Sharon and Lis, 1990). Legume lectin does not contain α-helix and therefore they belong to a class of β-proteins. Dimeric lectins are divalent whereas tetrameric lectins are tetravalent with four carbohydrate binding sites. Two monomers are associated to form a canonical dimer (fig.2). When the two dimers are associated through their back walls in which two monosaccharides binding sites occur at both sides of the tetramer, 222 (D2) symmetry is formed in the tetrameric molecule (Young and Omen, 1992; Sharma and Surolia, 1997). Hence, carbohydrate binding sites occur at four corners of this tetrahedron (fig.3). But a peanut agglutinin (fig.4) has neither 222 (D2) symmetry nor a four-fold symmetry (Banerjee *et al*, 1994).

Legume lectins also bind to hydrophobic molecules 1, 8-anilinonaphthalenesulfonic acid (ANS), 2,6-toludinylnaphthalenesulfonic acid (TNS), adenine and phytohormones like cytokinin. These hydrophobic binding sites are entirely different from the carbohydrate binding sites, e.g., Con A and binds porphyrins as well as certain peptides that act as carbohydrate mimetics (Kaur *et al*, 1997; Jain *et al*, 2000).



(Fig 1. Crystal structure of Con A. Blue colour indicates the di- Pink sphere indicates the Manganese atom).

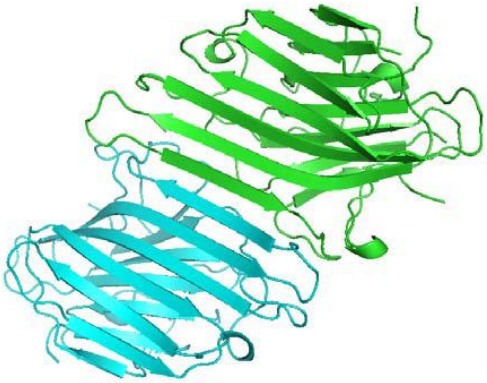


Fig 2. A dimer of Con A, two monomers are associated non- covalently to form an anti parallel β-sheet through inter molecular strands).

(Fig. 3 Quarternary structure of Con A)

b) Monocot mannose-binding lectins

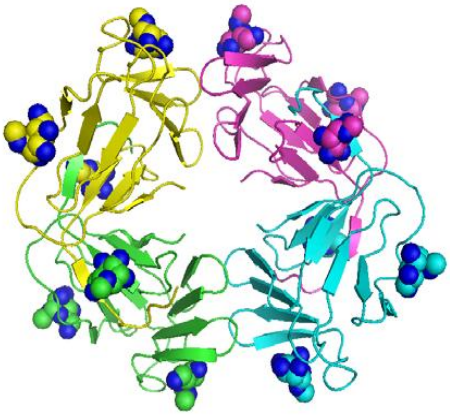
This lectin belongs to the sub-group of monocots. They are mannose-specific lectins and are present in monocot families like Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Liliaceae, and Orchidaceae (Van Damme *et al*, 1998). They occur mainly in leaves, roots, flowers, ovaries, bulbs, tubers, rhizomes, roots, nectar (Peumans *et al*, 1997) and rarely in seeds.

The first lectin to be crystallized and analyzed by X-Ray diffraction (Hester *et al*, 1995) was *Galanthus nivalis* agglutinin (GNA) and its 3D structure was determined. It has a β-prism II fold structure.

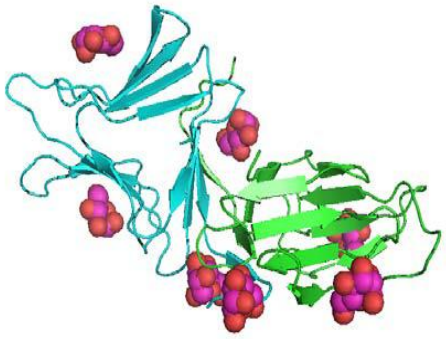
Structure

The monomer consists of three sub domains (I, II, III). Each domain consists of four stranded β-sheet. Each domain has a Carbohydrate Recognition Domain (CRD). These three sub domains form the three faces of a prism. They are connected by loops to form a 12-stranded β-barrel that form three mannose binding sites (fig.4) present in the clefts that are formed by three bundles of β-sheet, e.g., daffodils (Sauerborn *et al*, 1999), bluebells (Wood *et al*, 1999), etc. that recognizes mannose containing glycoprotein, hence showing inhibitory activity on the invitro replication of retroviruses (Balzarini *et al*, 1991 and 1992). While these lectins bind to the surface of glycoprotein on HIV with high affinity, the dimeric garlic lectin (fig.5) cannot bind (Vijayan and Chandra, 1999).

These lectins known as storage proteins are abundantly found in storage tissues. They protect the plants against sucking insects (Rahbe *et al*, 1995) and invertebrates (Hilder *et al*, 1995).



(Fig. 4 Crystal structure of GNA)



(Fig. 5 Crystal structure of dimeric garlic bulb lectin)

c) Chitin binding lectins

These proteins consist of one hevein domain. Chitin binding lectins are found in plant families like Gramineae, Solanaceae, Phytolaccaceae, Urticacea, Viscaceae and Papavaracaceae (Peumans *et al*, 1996; Raikhel *et al*, 1993). They occur mainly in seeds and vegetative tissues. They belong to different classes like merolectins, hololectins and chimerolectins. The examples of chitin binding lectins without hevein domain are Chitin binding legume lectins and Cucurbitaceae phloem lectins (Van Damme *et al*, 1998).

Structure

Hevein is a small 43 amino acid protein obtained from the latex of rubber tree (Waljuno *et al*, 1975). Hevein domain (Raikhel *et al*, 1993) is a structural unit of 40 amino acid residues that possess chitin binding activity. It is a typical merolectin. Its 3D structure has two short α-helices and a stretch of amino acid residues at the N-terminal end of polypeptide chains that form two strands of anti-parallel β-sheet followed by α-helix (fig.6). The structure of hevein is stabilized by four disulphide bonds.

Hololectins like monomeric lectins of *Urtica dioica* has two hevein domains (Peumans *et al*, 1984), dimeric Wheat Germ Agglutinin has four hevein domains (Reikhel *et al*, 1993). Each domain is folded into a compact globule through four disulphide bonds and 5-6 β-helices. Each domain has a carbohydrate binding site (Wright, 1990).

Chimeric lectin is of two types:

Class I chitinases

It has a N-terminal chitin binding domain linked to active chitinase domain (Collinge *et al*, 1993; Beintema, 1994). They help in plant's defence against fungi.

Class II chitinases

It consists of dimeric Solanaceae lectins. Its proteins are built up of chimeric polypeptides consisting of N-terminal chitin binding domain with three hevein repeats (Kieliszewski *et al*, 1994; Allen *et al*, 1996).

e.g., *Lycopersicon esculentum* agglutinin and *Solanum tuberosum* agglutinin.

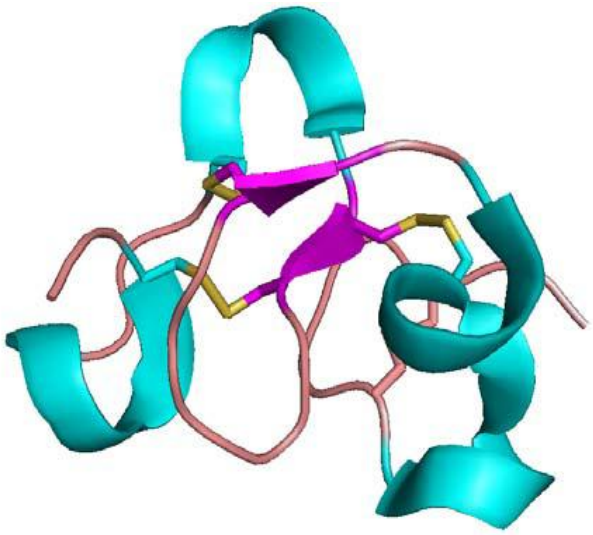


Fig.6 3D structure of hevein. Cyan colour indicates α-helices, magenta colour indicates β-helices, and yellow colour indicates disulphide bonds.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

Canavalia ensiformis commonly known as Jack bean belongs to the group of flowering plants belong to the legume family i.e., Leguminosae (or Fabaceae). It is a twinning plant of height 1 metre. Its deep roots make the plant drought resistant. Its foliage is edible and used as a fodder for animals and animal nutrition mainly in Brazil. It is mainly found in tropical regions. The chemicals produced by Jackbean provide defence to many organisms like caterpillar, etc. The lectin Concanavalin A is obtained from the Jackbean seeds. This lectin is widely used in industry and scientifically, it is used as a reagent in glycoprotein, biochemistry, immunology and in biotechnological applications i.e., lectin affinity chromatography. The purified form of enzyme-Urease that is used in scientific research is obtained from Jackbean.

3.1 Biological Classification:

Fig. 7 Jackbean plant

Fig.8 Jackbean seeds

Kingdom - Plantae

Subkingdom - Tracheobionta

Superdivision - Spermatophyta

Division - Magnoliophyta

Class - Magnoliopsida

Subclass - Rosidae

Order - Fabales

Family - Fabaceae

Genus - *Canavalia*

Species - *ensiformis*

3.2 Concanavalin A

Concanavalin A is a group of plant proteins called as lectins (Boyd, 1963). It is the first legume lectin that was purified and crystallized from the seeds of Jackbean (*Canavalia ensiformis*). It is a single-cell protein. It binds to the nonreducing terminal α-D-mannosyl and α-D-glucosyl groups of sugars, glycoproteins, and glycolipids (Goldstein and Poretz, 1986). It is a metalloprotein containing Mn^{+2} ions and Ca^{+2} ions. Under the influence of pH Con A can exist as a dimer or a tetramer (Loris et al, 1998). It can also exists as dimetallised dimer in two different forms i.e., in dimeric or tetrameric forms.

3.2.1 Characteristics of Concanavalin A

Con A is a 104 kDa protein (Sumnar *et al*, 2002) having four identical subunits. It can bind with the carbohydrates like α-D-glucose and α-D-mannose having unmodified OH groups at C-3, C-4, C-6, and terminal glucose residues of proteins and peptides. It can also agglutinate red blood cells by interacting with immunoglobulin glycopeptides. It is a lymphocyte mitogen. It can also binds with some bacteria. The metal ions present in Con A helps in binding and stabilizes its conformation. But the buffers, EDTA and other metal cholators loses its carbohydrate binding affinity. Concanavalin A is also a plant mitogen since it has the ability to activate mouse T-cell subsets that give rise to four T-cell populations such as precursor to suppressor T-cell, one subset of human suppressor T-cells sensitive to Con A (Powell and Leon, 1970).

3.2.2 Structure of Concanavalin A

Concanavalin A is a homotetramer each subunit (26.5 kDa) consisting of 237 glycated amino acids that bind to metallic ion i.e., Ca^{+2} or Mn^{+2} (Hardman and Ainsworth, 1972). The specific feature of its amino acid is it has two anti-parallel beta sheets in which one sheet consists of seven strands and another sheet has six strands. It has the affinity towards mannose and glucose mainly. It has four binding sites, in the four sub-units. Each of the sub-unit has one saccharide binding site and two metal binding sites i.e., transition metal (Kalb and Levitzki, 1968). In its native state, one molecule of Con A binds to one Ca^{+2} ion and one Mg^{+2} ion. Both pyranosides and furanosides bind to the same site of Con A. Its molecular weight is 104 to 112 kDa and isoelectric point is between 4.5 to 5.5. Concanavalin A has a low-frequency wave number of 20 cm in its Raman spectra. It can also initiate cell division on T-lymphocytes by stimulating the energy metabolism of thymocytes (Lei and Chang, 2009).

3.2.3 Biological activity of Concanavalin A

Concanavalin A can interact with carbohydrates containing mannose as well as it can interact with rhodopsin, immunoglobulins, lipoproteins and carcino-embryonary antigen. It has been found that tryptophanyl and tyrosyl residues are involved in the binding of carbohydrates (Moore and Mudher, 1979). Mainly, it can agglutinate red blood cells as well as various cancer cells (Betton, 2004). When the transformed cells and normal cells were treated with trypsin, they do not agglutinate at 4°C but they agglutinate only in the presence of Concanavalin A. A dimer is formed by joining two six strands. Two dimers then form a complex by layering twelve stranded sheets. As a result, four separate binding sites for oligosaccharides are formed and thus they are able to agglutinate cells like erythrocytes, myocytes, B-lymphocytes, fibroblasts, rat thymocytes, human fetal (Edelman and Millette, 1971), intestinal epithelial cells, adipocytes (Inbar and Sachs, 1969), etc. Con A can also interact with mannose residues of microbes like bacteria-E.coli, Bacillus subtilis and protist-Dictyostelium discoideum. It can stimulate several matrix metalloproteinases (MMPs).

3.2.4 Applications of Concanavalin A

Concanavalin A has many applications in many aspects:

It is used to immobilize huge quantities of glycoenzymes without losing their activity or stability. These non-covalent couplings of Con A-glycoenzyme can be reversed by adding sugars or decreasing its pH. Moreover, Con A also triggers autophagic cell death by binding to the cell

membrane of glycoproteins. It can also inhibit the growth of tumor nodule partially. It is useful for studying cell division, cell surface, immune regulation by various immune cells (Dwyer and Johnson, 1981), characterizing of glycoproteins, purifying glycosylated macromolecules in lectin affinity chromatography.

Concanavalin A also induces autophapy in the cell. When Con A binds to mannose moiety of the glycoprotein present on the plasma membrane, it is internalized to mitochondria. As a result, BNIP3-mediated autophagy is induced and the tumor cells undergo autophagic cell death. Moreover, Con A is also known as T cell mitogen since it can induce hepatitis in mice by triggering NKT cells and activating $CD4^+$ T cells. The anti tumor effect of Con A is mediated by the activation of $CD8^+$ T cells that establishes a tumor antigen-specific immune memory and inhibiting the tumor (Chang *et al*, 2007). Moreover, it also functions as an anti-hepatoma effect in the following manner:

When Con A enters into the liver, it binds to hepatoma cells through its specific binding property to the mannose residue present on tumor cell membranes and gets accumulated in the liver nodules and hepatic inflammation occurs thus inducing the hepatoma cells to undergo autophagy as well as it also activates the immune cells in the liver. Moreover, adaptive response is induced against the tumor that leads to liver tumor regression.

Initially, Con A has no effect on specific tumor cells but due to hepatic inflammation by its deposition, tumor antigens are processed and presented to tumor antigen-specific $CD4^+$ T cells and CD8+ T cells. After that, tumor-specific immunity is established.

Autophagy is a new target for cancer therapy if the molecules required to suppress tumorigenesis can be identified. It plays a protective role in maintaining homeostasis and controlling the quality of the protein and organelle of the normal cells. It mainly occurs under metabolic stress condition. In case of cancer cells, autophagy becomes crucial since cancer cells require high energy for their uncontrolled cell division (Lei and Chang, 2009).

Moreover, autophagy maintains cellular metabolism under starvation by removing the damaged organelles under stress thus improving the survival of the cells. But prolonged induction of autophagy leads to cell death.

Besides autophagy, chemotherapy-induced cell death process will also induce anti-tumor response. The association of a direct effect of cytotoxic drug and an indirect immune-mediated cytotoxic effect of Con A occurs due to its specific mannose binding property by which it induce autophagy on target cells and immune-modulate the lymphocytes. In this way, Con A has

emerged as a novel type endogenous cancer vaccine immunotherapy (Lei and Chang, 2009). The combined effect of direct induction of autophagy on target cells and indirect immunomodulating activity on lymphocytes by binding to mannose on tumor cells induces an *in-situ* inflammatory response and anti-tumor effect on cells. Due to this property of Con A, it act as an anti hepatoma agent as well as it is also a natural lectin in the form of anti-cancer compounds.

3.2.5 Toxicity of Concanavalin A

Concanavalin if utilized in excess amount can be harmful to the organisms. They can damage the intestinal mucosa. It is due to its toxicity that triggers apoptosis and autophagy in cell i.e., it is responsible for killing of cancer cells by inhibiting the activity of DNA polymerase alpha, thus blocking the further replication process and no more cell division occur. Concanavalin A does not agglutinate normal cells, but it has a high agglutination activity towards cancerous cells or transformed cells (Inbar and Sachs, 1969) due to the presence of their exposed binding sites for Concanavalin A, where it binds to glucose or mannose present on the cell membrane and agglutinates and resulting in cell toxicity, thus inhibiting tumor formation.

OBJECTIVES

4. OBJECTIVES

The main objectives include:

1. To isolate and characterize the protein of interest(Concanavalin A) from the Jackbean seeds by affinity chromatography,
2. To determine the concentration of protein,
3. To determine their agglutination activity by Hemagglutination Assay,
4. To determine their molecular weight by SDS-PAGE.

MATERIALS AND METHODS

5. MATERIALS AND METHODS

5.1 Sample Collection

The dry Jackbean seeds were collected from the Department of Biotechnology in Indian Institute of Technology, Kharagpur and 1 ml of blood was collected from CWS Hospital, Rourkela.

5.2 Chemicals

Acrylamide, Bisacrylamide, Sodium dodecyl sulphate (SDS), Ammonium persulphate (APS), N,N,N',N'-tetramethylenediamine (TEMED), β metacarpoethanol, Bovine serum albumin(BSA), Tris were purchased from Sigma Aldrich, USA. Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3), Potassium Sodium Tartarate ($KN_aC_4H_4O_6$), glycine, Copper Sulphate ($CuSO_4$) was purchased from SRL, Cisco Research Laboratories Pvt. Ltd., Mumbai. Folin-Ciocalteau phenol reagent, Potassium hydrogen phosphate (K_2HPO_4) and Potassium Dihydrogen Phosphate (KH_2PO_4) were purchased from S.D. Fine Chem. Ltd., Mumbai. Bromophenol blue, Acetic Acid, Agarose were purchased from Himedia, Mumbai. Glycerol was purchased from RANKEM Pvt Ltd., Pre stained molecular weight marker was purchased from Bio-Rad, India, Methanol, Silver Nitrate, Sodium Thiosulphate were purchased from Nice chemicals Pvt. Ltd., India, Ethanol was purchased from Trimurty Chemicals, India.

5.3 Preparation of Maltamyl Affinity Sepharose 4B matrix

Maltamyl Sepharose 4B affinity matrix was prepared by the following methods.

5.3.1 Epoxy activation of Sepharose 4B

8g of Sepharose 4B was suspended in 12 ml of water and matrix was separated from it powder form and diluted with Phosphate Buffer Saline (PBS) negative succisor. Alcohol is used to preserve Sepharose 4B. 4g of Maltamyl Sepharose 4B was washed with 6ml distilled water. Then 2.6 ml of 2N Sodium Hydroxide was added to it followed by the addition of 0.65 ml of epichlorohydrin. Then they were incubated at 40°C for 2 hours. Then PBS along with 8g Sodium Hydroxide and distilled water (100 ml) was added to it. Matrix was separated from Sepharose 4B in powder form and treated with PBS. Then 6ml of water was added to 4g of Sepharose 4B and transferred to glass filter funnel and the gel was washed with 500ml distilled water.

5.3.2 Preparation of Amino Sepharose 4B

6 ml of concentrated ammonia was added to it and then washed with water again.

5.3.3 Coupling of lactose with amino Sepharose 4B

Suction dried Sepharose 4B was suspended in 3 ml of 0.2M di-potassium hydrogen phosphate buffer containing 51mg maltose and incubated at room temperature for 10 days with occasional shaking.

5.4 Isolation and purification of Concanavalin A

Con A was extracted from 50g of jack bean seeds by soaking them in 150ml of PBS solution overnight and was grounded. The total weight of paste was found to be 220.20g. Then they were centrifuged at 7000 rpm for 20 minutes at 4°C. The supernatant obtained from homogenous paste was crude. Then 30% cut-off was made in 50ml of supernatant by saturating them with 8.2g of ammonium sulphate and stored at 4°C for 12 hours. Then they were centrifuged at 7000 rpm at 4°C for 20 minutes. Pellet was discarded and 90% cut-off was made with 45ml of the supernatant by saturating them with 18.09g of ammonium sulphate and stored at 4°C overnight. The next day the increased volume to 50ml was centrifuged at 7500 rpm at 4°C for 20 minutes. The pellet was mixed with 20ml distilled water and vortexed and made upto 35ml. These samples were kept for dialysis in distilled water for 2 days and in PBS for 1 day. Then 40ml of the sample centrifuged at 7500 rpm at 4°C for 20 minutes and 48ml of supernatant was collected and stored at 4°C.

5.5 Affinity Chromatography

The maltamyl sepharose beads were washed in PBS solution its O.D. was measured at 280 nm. Then 38ml of protein sample was loaded on the maltamyl sepharose beads and O.D. of the flow through was measured at 280 nm. Then the maltamyl sepahrose beads were again washed with PBS solution to remove the undesired proteins. 50ml of 4M maltose solution was loaded on the Maltamyl Sepharose 4B beads and O.D. of the eluent was measured at 280 nm. Since the maltose sugar are bounded to eluent sample, so they can be removed by dialysis in 2 litres of PBS solution and stored at 4°C for 1 day.

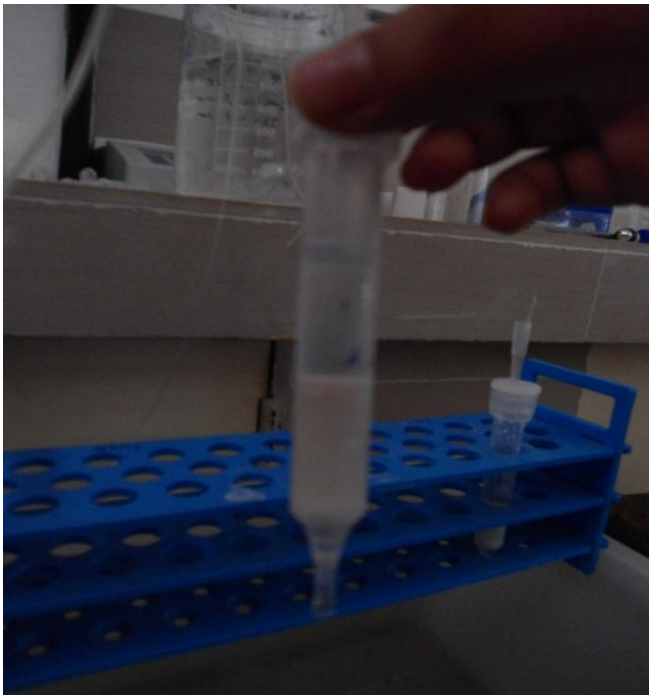


Fig.9 Affinity Chromatography

5.6 Estimation of Protein Concentration by Lowry method

REAGENT A - Sodium hydroxide (0.5%),

Sodium carbonate (2%) upto 1 litre,

REAGENT B1 - 1% Copper sulphate,

REAGENT B2 - 2% Sodium Potassium Tartarate,

REAGENT C - A: B1:B2 = 100:1:1,

BSA STANDARD - 1mg/ml,

Folinciocalteau's reagent - 1N (5 ml solution +5 ml distilled water).

Different concentration of BSA solution were taken from stock solution and distilled water was added to it and made up to 2ml. Unknown quantity of Con A protein was dissolved in 1ml distilled water and 5 ml of reagent C and 0.5ml of protein was added to it and mixed properly. Then they were incubated for 10 minutes. Then 0.5 ml of Folin reagent was added and incubated for 30 minutes. Their O.D. was taken at 750 nm. While doing this, it failed, so their O.D. was taken directly at 750 nm.

Concentration of protein was determined by 50 times dilution of crude and 30% sample (40µl sample + 1960µl PBS), 20 times dilution of 90% sample (100µl sample + 1900µl PBS) and affinity sample (2000µl sample) was measured directly. Then their O.D. was measured at 280 nm.

5.7 Normalization of protein sample

After measuring their O.D., the diluted sample was normalized in the following manner:

Crude sample (550µl sample + 1450µl PBS)

30% sample (900µl sample + 1100µl PBS)

90% sample (1.6ml sample + 400µl PBS)

Affinity sample (without dilution)

The above normalized samples were then stored at 4°C.

5.8 Haemagglutination Assay

Haemagglutination activity of Concanavalin A was detected by using human erythrocytes in the presence of anti-coagulant. 1 ml of the blood sample was centrifuged at 1000 rpm for 5 minutes. Then 10ml of PBS was added to the pellet and was again centrifuged at 1000 rpm for 5 minutes. Then 100µl of the pellet was collected and dissolved in 10ml of PBS. The protein sample was serially diluted in microtiter plate that was further mixed with 100µl of the human erythrocytes. The first well serve as positive control since it contain 100µl of normalized sample and 100µl of blood. Last well serve as negative since it contain 100µl of blood and 100µl of PBS solution.

5.9 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according by method of 12% acrylamide gel. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) by preparing stacking gel(5%), resolving gel(12%), electrode buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, gel loading buffer (5x) containing 50 Mm Tris-Cl (p^H 6.8), 100 mM β-mercapto ethanol, 2% (w/v) SDS, 10% (v/v) glycerol and 0.1% bromophenol blue. Proteins in the sample buffer were boiled for 3 min at 90°C before electrophoresis. Electrophoresis was carried out until the bromphenol blue marker reached the bottom of the gel. The stacking gel was run at 90v and resolving gel at 140v.

Silver Staining

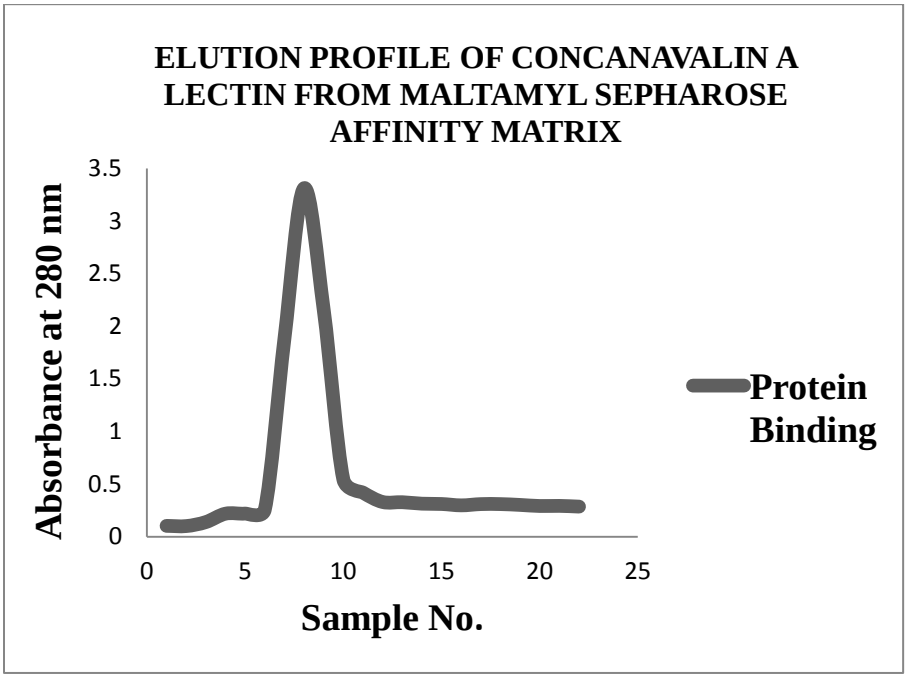
Silver Staining was done on gel for the localization and visualization of the band of protein sample. Fixing of SDS-PAGE gel was done with fixing solution (50ml) containing methanol (25ml), acetic acid (6ml), formaldehyde (25µl) and rest water for 1 hour. Then it was washed by 50% ethanol with shaking (3 times) for 10 minutes each time. Then it was pre-treated with Sodium Thiosulphate (0.01%) for 1 minute and rinsed with double distilled water 3 times for 20 seconds each time. Then the gel was impregnated with Silver Nitrate solution (0.06g) and formaldehyde (20µl) dissolved in 30ml water for 20 minute and then rinsed with distilled water 2 times for 20 seconds each time. Then the gel was treated with developing solution (30ml) containing Sodium Carbonate (1.8g), 1 Sodium Thiosulphate crystal and formaldehyde (15µl) and was shaken for 10 minutes till the bands are developed and then rinsed with distilled water 3 times for 20 seconds each time. The gel was then treated with Stopping solution or fixing solution (50ml) for 10 minutes. Protein bands were observed on the gel and the gel was then put on the gel doc to determine its corresponding molecular weights.

RESULTS AND DISCUSSION

6. RESULTS AND DISCUSSION

6.1 Purification of Concanavalin A

The Jackbean extract was precipitated to 30% followed by 90% precipitation by Ammonium Sulphate fractionation method. Then they were dialysed for 3 days. After that, the dialysed supernatant was stored at 4°C and then the maltamyl sepharose 4B beads were loaded with the dialysed supernatant followed by the 0.4M maltose solution. The unbound proteins were drained out during the washing of maltamyl beads with PBS solution at each interval. The protein that was bound to the maltamyl sepharose beads containing transition metal ions (Ca^{+2} or Mg^{+2}) ions were eluted by 0.4M maltose solution. These eluted protein solution had maltose bound on its surface since the protein has greater affinity to maltose sugar than maltamyl beads. Maltose was removed from the protein by dialysis of the eluent in PBS solution (pH 7.2) for 1 day. Thus, the purified form of protein was obtained through methods of the purification of Con A obtained from Jackbean seeds.



(Fig. 10 Elution profile of Concanavalin A lectin from maltamyl sepharose affinity matrix)

The above graph indicates that absorbance is maximum at 3.312 A°. This indicates that at

3.321 A°, the binding of protein to maltose sugar is maximum i.e., the protein of interest is maximum at that point.

6.2 Determination of protein concentration

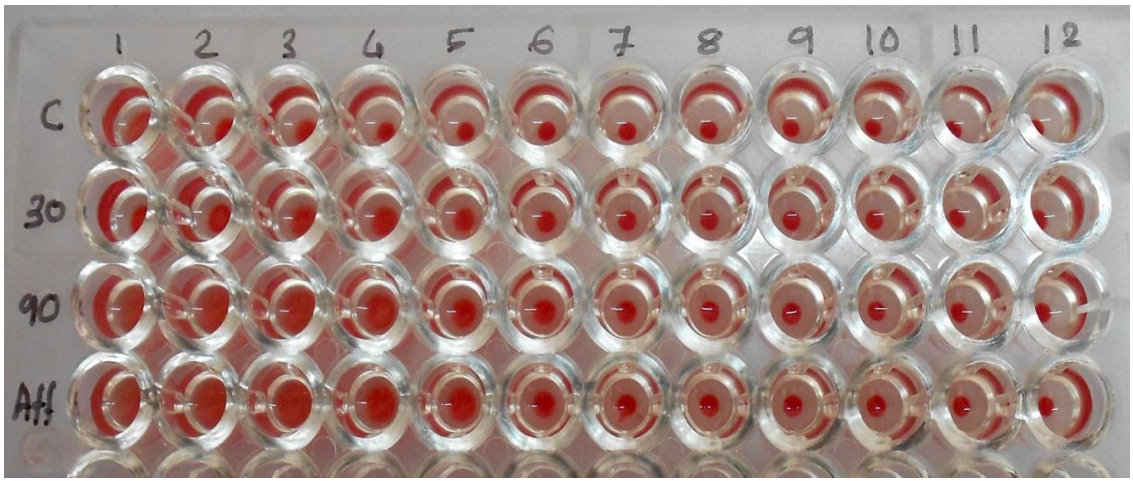
Table 1. Determination of protein concentration

Sample	Volume(ml)	O.D. at 280 nm	Concentration(mg/ml)	Total protein(mg)
Crude	50	46.6	37.28	1864
30%	45	31.9	25.52	1148.4
90%	38	27.1	21.68	823.84
Affinity	45	0.402	0.321	14.445

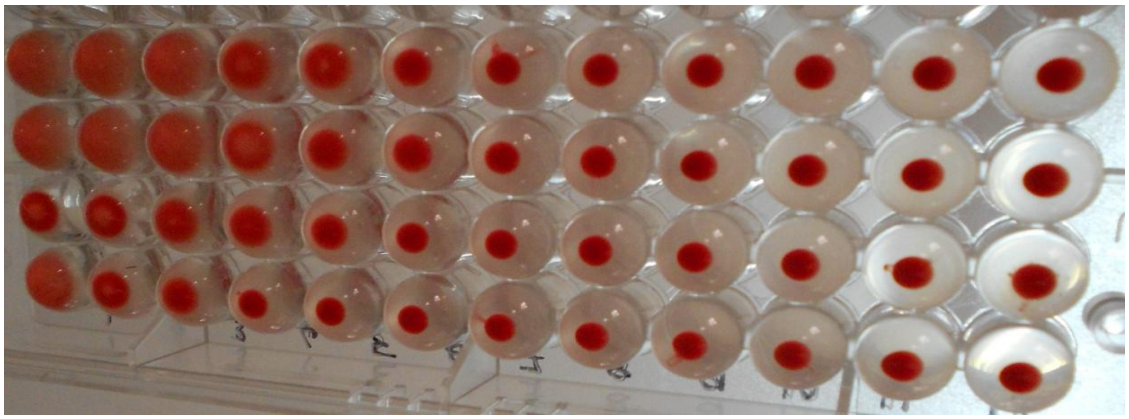
The above table indicates that concentration of protein is maximum in crude sample i.e., it contains various types of protein. But the concentration of proteins decreases due to the purification of the protein of interest. Since the main objective is to isolate and purify the protein of interest from Jack bean extract, so their concentration decreases and minimum in affinity. From this, it has been found that the most purified form of the protein (Concanavalin A) to be isolated is found in affinity sample.

6.3 Hemagglutination Assay

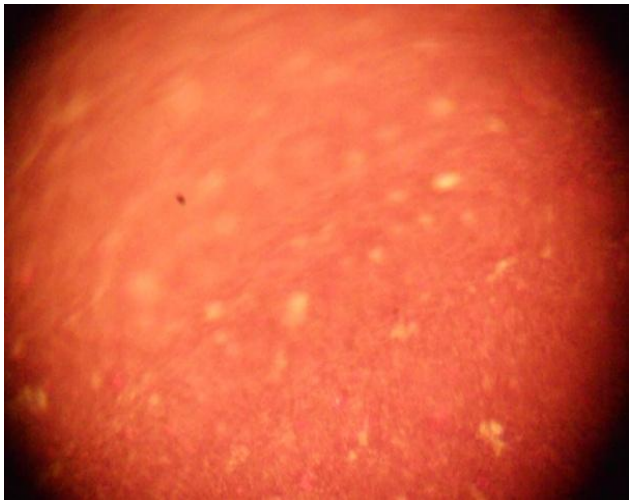
The agglutination activity of the normalized sample of Jackbean was tested with the human RBC. The affinity purified protein sample obtained by maltamyl sepharose 4B beads exhibit higher titer (2^2) of agglutination activity than that of crude, 30% and 90% protein sample. This titer value corresponds to Con A purified by Sephadex G50. Hence, the purified form of protein is a lectin and may be Con A.



(Fig. 11 Agglutination activity of proteins observed in microtiter plate from the top)

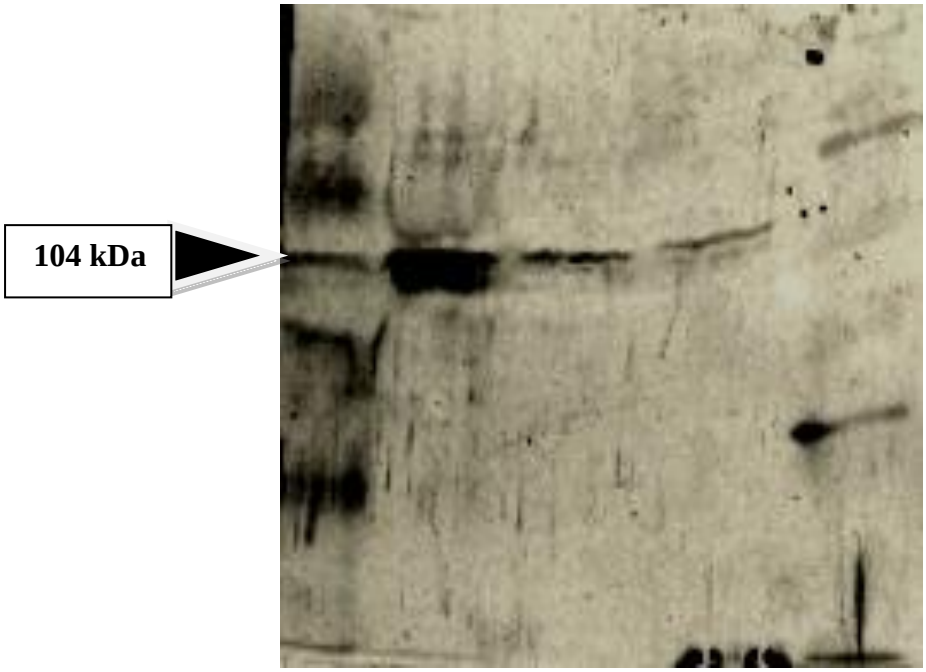


(Fig. 12 Agglutination activity of proteins observed in microtiter plate from its bottom)



(Fig. 13 Agglutination activity observed in closed view)

6.4 SDS-PAGE



(Fig.14 SDS gel showing bands of corresponding molecular weight after silver staining)

The protein sample was run into SDS-PAGE and band was observed after Silver Staining. Lane 1 to Lane 5 represents the crude, 30%, 90%, affinity sample and pre-stained molecular weight marker respectively. A clear distinct band was observed that corresponds to 104 kDa in the affinity portion. This molecular weight of 104 kDa corresponds to the molecular weight of commercially obtained Concanavalin A. Hence, it has been confirmed that the protein of interest that was isolated and obtained in purified form by the above methods was Concanavalin A.

CONCLUSION

7. CONCLUSION

The yield of purified form of Concanavalin A by affinity chromatography through maltamyl sepharose 4B beads was higher than those obtained by Sephadex G-50 by batch process. This purified form of isolated lectin was identical to Concanavalin A in all aspects as determined by SDS-PAGE, Haemagglutination Assay and purification through maltamyl sepharose 4B beads through 0.4M maltose sugar. Concanavalin A has a greater affinity towards maltose sugar than the maltamyl sepharose 4B beads. So, maltose sugar is necessary to get a purified form of Concanavalin A in which it binds to maltose sugar. Moreover, Concanavalin A has a greater agglutination activity with human erythrocytes and was found that its titer value is 2^2. From SDS-PAGE, it has been found that the molecular weight is 104 kDa. Thus, it was confirmed that the isolated protein was Concanavalin A.

Since the cost of maltamyl sepharose 4B beads is very low in comparison to the cost of Sephadex G-50 used for the Concanavalin A purification method, hence the purification of Concanavalin A by maltamyl sepharose 4B beads is preferred in terms of storage for a longer days, higher yield of protein and low cost.

FUTURE PROSPECTS

8. FUTURE PROSPECTS

Further research can be carried out on Concanavalin A in various cell lines i.e., cancer cell lines and has made a great contribution to the animal cell glycobiology. Concanavalin A has its significance in various ways. It triggers the apoptosis and autophagic pathway. It has a therapeutic effect on hepatic cancer cells. It is also used for the immobilization of enzymes. Moreover, Con A forms the basis for the study and treatment of retinal diseases in future.

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ABBREVIATIONS

PBS: Phosphate Buffer Saline

et al: And others

Rpm: Rotation Per minute.

Conc: Concentration

Hrs: Hours

L: litre

Mg: milli gram

pH: Hydrogen concentration

NaOH: Sodium hydroxide

Na_2CO_3 : Sodium carbonate

APS: Ammonium per Sulphate

TEMED: N,N,N',N'-tetramethylenediamine

KNaC4H4O6: Potassium Sodium Tartarate

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

BSA: Bovine serum albumin

KH2PO4: Potassium dihydrogen Phosphate

K2HPO4: Potassium hydrogen phosphate

(NH4)2SO4: Ammonium Sulphate

Pvt .Ltd: Private limited

kDa: kilo dalton